The Formation of the Anthocyan Pigments of Plants. Part IV.*—*The Chromogens.*

By Frederick Keeble, Sc.D., Professor of Botany, University College, Reading; E. Frankland Armstrong, D.Sc., Ph.D.; and W. N. Jones, M.A., Lecturer in Botany, University College, Reading.

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The object of the series of communications of which the present paper forms a part is the elucidation of the biochemistry and genetics of flower-pigmentation. In order to achieve this object it is necessary, in the first place, to ascertain the nature of the chemical processes which determine the formation of the anthocyan pigments, and, in the second place, to discover the chemical nature of the Mendelian characters to which the several varieties of a given species owe their power of forming and breeding true to definite types of flower colour.

The history of the working hypothesis which we use in these investigations has been summarised in an earlier paper.† This hypothesis may be expressed in the form of the equations A and B:—

A. Prochromogen + enzyme = chromogen.
? a glucoside. ? emulsin.

B. Chromogen + oxydase = anthocyan pigment.
Peroxydase + organic peroxide.

Our previous and present communications are concerned with the latter, consideration of equation A being reserved for a subsequent occasion.

The position to which our previous work has led us may be summarised thus:—

The presence of oxydase in flowers may be demonstrated by means of benzidine, α-naphthol, or similar "artificial chromogens," which, when acted

* Previous papers of the series, which did not bear the general title, are:—

[Part I.]—"The Distribution of Oxydases in Plants and their *Rôle* in the Formation of Pigments," 'Roy. Soc. Proc.,' 1912, B, vol. 85; [Part II.]—"The Oxydases of *Cytisus Adami*," 'Roy. Soc. Proc.,' 1912, B, vol. 85; [Part III.]—"The *Rôle* of Oxydases in the Formation of Anthocyan Pigments of Plants," 'Journ. Genetics,' Nov. 1912, vol. 2, No. 3.

† "The *Rôle* of Oxydases in the Formation of Anthocyan Pigments of Plants," 'Journ. Genetics,' 1912, vol. 2, No. 3.

on by oxydase, yield pigments. These reagents serve not only to demonstrate the occurrence but also to determine the distribution of the oxydases of the flower. By the application of the method it is found that the distribution of oxydase coincides with that of anthocyan pigment. In white flowers oxydase may be present in an active or an inhibited state. In the former case some other part of the pigment-forming mechanism is absent from the flower; in the latter, the whole of that mechanism is present, but its action is prevented by the inhibition of the oxydase.

The present communication and that which follows (Part V) deal primarily with the chromogens of the flower.

Our first definite results demonstrating the existence of chromogens in the flower and the relation of these colourless substances with the anthocyan pigments were obtained with Brompton Stocks (*Matthiola incana*). These plants occur in numerous colour-varieties, the chief of which are pink, red, purple, and purple flaked with white.

Flowers of any of these varieties, when treated with alcohol, lose their colour rapidly. It is therefore easy to obtain a series of colourless petals derived severally from each of the colour-varieties.

If the decolorised petals of such a series be placed in water at room temperature they begin almost at once to regain their colour, and, after a quarter of an hour, each petal is found to be possessed of the identical colour of the variety to which it belongs. The petal originally pink recovers its pink colour, that from a red or purple variety becomes again red or purple, and that from a white-striped purple variety reproduces with faithful accuracy the purple and white pattern of the original.

Despite the fact that our experiments have been carried out during the winter months, when suitable material is somewhat scanty, we have been able to prove that a similar recovery of natural colour is exhibited by many other flowers, for example :—

Aubretia, wallflower (Cruciferæ).
Viola (Violaceæ).
Pelargonium (Geraniaceæ).
Cyclamen, polyanthus (Primulaceæ).
Begonia (Begoniaceæ).
Azalea (Ericaceæ).
Bilbergia (Bromeliaceæ).
Dendrobium (Orchidaceæ).

Recovery of colour is shown also by the vegetative parts of plants which contain anthocyan, for instance, the leaves of Fuchsia and Tradescantia, and

the fronds of the Royal fern (*Osmunda regalis*). Loss and recovery of colour are therefore phenomena of very general occurrence, and may be regarded as characteristic of many, if not of all, kinds of anthocyan pigments.

The wallflower is of special interest in this connection, in that the brown varieties with which we have worked contain representatives of the two types of pigment—anthocyanic and plastid-derived pigments—to either or both of which the colour of flowers may be due. The brown colour of the wallflower is produced by a purple anthocyan pigment and a yellow plast pigment. When acted on by alcohol a brown petal becomes decolorised; but it recovers to a purple colour when treated with water. The recovery to purple instead of brown is due to the fact that the yellow plastid-pigment which contributes to the original brown colour is soluble in alcohol and is therefore extracted from the tissues by this reagent. Thus only the colourless antecedent of the purple anthocyan pigment is left in the cells. Treated with water that antecedent gives rise to a purple pigment which, since it is no longer mixed with yellow, produces its proper optical effect. The yellow pigment may be obtained free from the anthocyan pigment by evaporating the alcoholic solution and washing the residue with water, in which the plast pigment is insoluble. The power of recovering to the original colour serves as a means of distinguishing the pigments of the anthocyan class from those which are derived from the plastids.

The reproduction of the original colour in the petals of stocks and other plants is open to two alternative interpretations. On the one hand, it may be regarded as a phenomenon of like nature to that exhibited by indicators; on the other hand it may be attributed to the oxidation of a chromogen.

Immediate choice between the two interpretations is rendered difficult by reason of the fact that acids and alkalis exercise marked and definite effects on the colours of the anthocyan pigments contained in the flowers. Thus, in the presence of alkalis the pigment in the petals of stocks assumes a green-blue colour and in the presence of acids it becomes pink. Moreover the chromogen extracted by means of 50-per-cent. alcohol from the petals of stocks behaves as a very sensitive indicator. Dilution of the alcoholic extract with ordinary distilled water—which contains carbon dioxide—suffices to produce a pale pink colour. With mineral acids the colour becomes intense and with alkalis it passes through blue and blue-green to green.

Nevertheless, and in spite of the complication introduced by these indicator effects, the evidence of the experiments now to be described points very definitely to the conclusion that the indicator hypothesis must be discarded in favour of its alternative.

The petals of stocks decolorised by strong alcohol contain oxydase. The reproduction of the original colour by the immersion of decolorised petals in water is hastened by the addition of hydrogen peroxide. When a pink and a purple petal decolorised in the same alcohol are transferred to water to which a drop or two of hydrogen peroxide is added, the petals recover pink and purple respectively. Therefore it follows that the activating action of the peroxide is due to its provision of oxygen and not to its acidity.

In water which has been boiled for a long time in order to remove the oxygen, the recovery of colour, if it take place at all, occurs more slowly than in unboiled water. The addition of dilute hydrogen cyanide—a substance known to inhibit oxydase-action—prevents the recovery of colour. The results of other experiments designed to decide between the indicator and oxidation hypotheses lend further support to the latter. Thus decolorised petals in which the pigment has been caused to reform may be again decolorised either by leaving them in water till the pigment has diffused away or by transferring them to alcohol. Petals treated in this manner, if placed in hot water, produce once again their natural pigments.

Again, the restoration of pink colour to decolorised petals of a pink variety may be brought about in an alkaline medium; for example by transferring the petals from alcohol to water containing a small quantity of hydrogen peroxide which has been rendered faintly alkaline. The pink colour which is thus induced changes subsequently to purple. Conversely the purple colour returns to a petal of a purple variety even though the medium in which the change is effected be acid. In this case the recovered purple soon becomes pink owing to the action of the acid.

The last experiment is rendered still more conclusive if the procedure be modified in the following manner. Petals of purple stocks are incubated with 99-per-cent. alcohol to which enough citric acid has been added to render the alcohol distinctly acid to litmus. The petals became almost decolorised, retaining only a faint pink colour. When transferred to distilled water—which is not acid to litmus—pigment is produced in considerable quantities and the colour of the pigment is at first red but soon becomes purple. If the colour were of the type of an indicator reaction the effect of the water would be not to intensify but to dilute the tint.

The conclusion which we have reached as the result of these observations is that, although indicator changes run parallel with the changes involved in the formation of anthocyanic pigments, the latter arise as the result of the oxidation of chromogens.

It remains to mention the remarkable acceleratory effect which high temperature has on the reproduction of the natural pigments of stocks. As

stated already, a petal recovers its colour in water at room temperature in the space of a quarter of an hour. At higher temperatures the recovery is more rapid and if a petal be dropped into water which has been heated to near the boiling point the recovery of colour is almost instantaneous.

We turn now to the detailed interpretation of the facts of loss and recovery of colour; and we deal first with the loss of colour which takes place when petals are dehydrated.

The evidence about to be given supports the conclusion that the loss of colour is due to the action of a reducing agent. In the present state of our knowledge of the reducing processes which occur in the cells of plants it is not possible to affirm that the agents of these processes are of the nature of specific catalysts. We propose therefore to avoid using the word reductase and to employ the indifferent term "reducing agent" in the description of the phenomena of decolorisation.

A careful examination of the petals of stocks subjected to the action of alcohol makes it difficult to escape from the conclusion that decolorisation is due to the activity of a reducing agent. It is easy to demonstrate that the loss of colour is not due merely to a dissolution of the pigment and its diffusion throughout a large bulk of fluid.

As evidence that the loss of colour is due to the action of a reducing agent we may cite the following facts:—

The immersion of the petals in alcohol produces three immediate effects,—a rapid evolution of gas, a reduction in the amount of colour, and a discharge from the petals of a certain amount of pigment which dissolving in the alcohol gives rise to a marked coloration of that reagent.

Similar effects are produced, but more rapidly, if previously to their immersion in alcohol the petals are treated for about half a minute with chloroform.

As a consequence of the discharge of the pigment the alcohol becomes deeply coloured—purple or red according to the variety of stock used in the experiment. If the alcohol be decanted at once its colour disappears with remarkable rapidity and in less than 5 minutes the liquid becomes colourless or at most faintly coloured. The partly decolorised petals, from which the first lot of alcohol was removed, if treated with more of this reagent, undergo further decolorisation, but at a much slower rate. The simultaneous evolution of gas and the discoloration suggest that the effect of the alcohol is to liberate a reducing agent which brings about the deoxidation of pigment and an evolution of oxygen. Further evidence of the presence of such a reducing agent is provided by extracts prepared by pounding fresh petals with alcohol. The colour of the extract is at first

identical with that of the petals from which it was made; but sooner or later the colour fades and the solution becomes colourless. The fading is rapid in concentrated alcohol and slow in alcohols of somewhat weaker grades. The agent responsible for the fading is resistant to high temperatures. Thus if alcoholic extracts be evaporated to dryness and the residues be taken up with water, the fading of the solutions still takes place Further evidence in favour of the view that decolorisation is due to reduction is offered by the results of experiments on the effect of extracts in inhibiting and in reversing oxydase-action.

The experiments were made in the following ways :—

1. *Extracts made from Stocks by Grinding the Petals with Alcohol.*—A solution of the peroxydase of bran is rendered of such a strength that it just gives the characteristic blue reaction with benzidine and hydrogen peroxide. Petals of a coloured variety of stock are ground with alcohol, the extract is evaporated to dryness, and the residue dissolved in water. If a few drops of the latter solution be added to the solution of peroxydase, and if the benzidine-hydrogen peroxide test be applied, no colour-reaction ensues. The oxydase is prevented by the reducing agent from bringing about the oxidation of benzidine. Only if it be increased very considerably in amount is the oxydase able to overcome the opposing influence of the reducing agent, and to bring about the oxidation of the benzidine.

2. *Extracts obtained by Immersing Intact Petals in Strong Alcohol.*—The use of extracts made by grinding petals with strong alcohol is open to obvious objections. We have, therefore, used extracts obtained by the immersion of intact petals in strong alcohol.

For this purpose petals of purple stocks are immersed in alcohol of 99 per cent. When the alcohol is decanted from the tube containing the petals, its colour (pale purple) disappears in the course of a few minutes. On evaporation over a water-bath it yields a purple residue. For the purposes of control an equal volume of alcohol of the same strength as that used for the extraction of the petals is also evaporated to dryness. A bran peroxydase is prepared of such a strength that when a given volume of it is added to a given volume of a weak solution of benzidine containing one drop of hydrogen peroxide a definite but pale blue colour is produced. The addition of similar volumes of peroxydase, hydrogen peroxide and benzidine to the purple residue results in the production of no blue colour, whereas the colour develops normally when the reagents are added to the vessel in which the alcohol alone has been evaporated to dryness. The alcohol which has been in contact with the petals, like the alcoholic extract obtained by maceration, prevents the action of oxydase.

Yet more conclusive is the result when the blue solution, produced by the action of bran peroxydase and hydrogen peroxide on benzidine, is added to the residue obtained by evaporating alcohol which has been in contact with intact petals. The blue colour of the former is discharged immediately, that is to say, the action of the oxydase is reversed, and the blue product of the oxidation of benzidine is reduced to its original colourless state. That this effect is not due to reducing agents present in the alcohol is shown by the fact that no discharge of colour is brought about by the addition of the blue oxydase-benzidine solution to the residue left after the evaporation of alcohol which has not been in contact with petals. This method of demonstrating the presence of a reducing agent is the more conclusive in that whereas alcohol alone reduces oxydase-activity, it does not bring about a reversal of the action. The only effect of alcohol on the blue colour is to precipitate the blue pigment.

We have shown in a previous communication (Part III) that the oxydases of the flower not only act on the forerunners of pigment contained in the petals but also on the artificial chromogen benzidine and give rise to pigments; we now show that flowers contain reducing agents which are not only capable of inhibiting the action of oxydase, but are able also to reduce both the natural pigments of the flower and the "artificial" benzidine pigments to the colourless state of chromogens.

Two facts stand out prominently in the foregoing investigation of decolorisation. These facts are that the reducing agent is very resistant to high temperatures and that it is active in strong alcohol. The former we have studied in sufficient detail only to be able to state that the reducing agent is not destroyed by exposure to a temperature of 100° C., the latter fact has been investigated more fully and with the following results:—

Both evolution of gas and fading of the flower take place rapidly in alcohol of 95 per cent. These processes go on, albeit more slowly, in yet stronger alcohol. Thus with the ordinary absolute alcohol of the laboratory (99 per cent.) a certain amount of gas is evolved and colour begins to disappear; but when petals are placed in alcohol of approximately 100 per cent. both processes, although they take place, come to an end much sooner than in the alcohol of slightly lower grade.

We conclude, therefore, that the reducing agent which brings about decolorisation of the petals of stocks is able to exhibit its specific action in tissues which are almost completely dehydrated. We have, moreover, evidence that loss of colour occurs naturally in the plant. We know, for example, that in many plants light shades of colour are dominant to dark shades; we know also that the flowers of such plant of stocks may assume as

they fade a new colour, and we know that the colour of some flowers undergoes a marked change during the course of the day. Such changes are to be ascribed to the simultaneous presence in the petals of pigments, chromogens, oxidizing and reducing agents.

We have now to consider the conditions under which recovery of colour occurs.

Evidence has been given already in favour of the interpretation that recovery is brought about by the oxidation of chromogen.

There is, however, one series of facts, namely, those bearing on recovery of colour by petals immersed in strong alcohol, which seems to throw doubt upon this conclusion.

For, as several investigators have shown, increasing concentrations of alcohol exercise a progressively adverse effect on enzyme action. Thus, Hudson (1910),* working with invertase has expressed the effects of different concentrations of alcohol in the form of a regular curve. He finds that in 70-per-cent. alcohol invertase retains only 10 per cent. of its activity.

We have studied the relation of the activity of maltase to alcoholic concentration and find that this enzyme is even more sensitive to ethyl alcohol than is invertase; the activity of maltase ceasing in 60 per cent. With methyl alcohol a 40-per-cent. solution suffices to render the enzyme inactive.

Our experiments with emulsin, which confirm those published in 1912 by Bourquelot, give results similar to the foregoing except in one important particular.

The activity of emulsin falls rapidly as the concentration of alcohol increases to 40 per cent. After this point is reached the activity falls off more slowly and some activity may be detected in solutions containing 90 per cent. of alcohol; in solutions containing from 40 to 90 per cent. of alcohol the activity of emulsin is proportional roughly to the amount of water present in the solution.

For the purpose of investigating the effect of alcohol on oxydase we have made use of the Lovibond tintometer. We measure by means of this apparatus the depth of coloration—a mixture of red and yellow—produced by the action of bran peroxydase on guaiacol.

The curve representing the amount of oxydase action—as measured by the tintometer—is similar to the curves which have been obtained for invertase, maltase and emulsin. As the alcoholic strength increases the activity of oxydase falls. In 50-per-cent. solutions it becomes very small and ceases altogether in 70-per-cent. alcohol. Alcohol causes a similar retardation of

* Hudson, C. S., 'U.S. Dept. of Agric., Bureau of Chemistry, Circular 58.'

the benzidine reaction; but the colour of the latter is not suitable for tintometric estimation.

The results obtained in test-tubes are opposed to the view that the recovery of colour in petals immersed in strong alcohol is due to the activity of oxydase. General considerations, however, led us to suspect that although alcohol of 70–80 per cent. may prevent the action of the oxydase in solutions extracted from plant-tissues, it might prove less potent to retard the action of oxydases in the tissues themselves. We have confirmed the truth of this suspicion in the following way:—

Petals of purple stocks were incubated with 99-per-cent. alcohol and when decolorised they were placed, some in 70 per cent., and others in 80, 90, and 95-per-cent. alcohol. Equal quantities of a solution of benzidine in water-free alcohol were added to each tube containing the petals, one drop of hydrogen peroxide was introduced into each tube, and the preparations were placed in the incubator at 37° C. Examination of the petals after half-an-hour showed that the petal treated with 70-per-cent. alcohol gave a well marked brown benzidine reaction for oxydase, the petal in 80 per cent. showed a very distinct reaction, that in 90 per cent. an equally good or even better reaction, and that in 95 per cent. a slight but distinct reaction in the veins of the claw.

Whence it follows that the peroxydase of stock petals is capable—if peroxide be present—of bringing about the oxidation of benzidine even in a medium containing 95 per cent. alcohol; and we infer that what is true of this artificial chromogen is true of the natural anthocyanic chromogen, namely, that the latter may undergo oxidation even in the presence of 95-per-cent. alcohol.

Thus the conclusion is reached that, although the experiments with oxydase extracted from plant tissues are adverse to the view that recovery of colour is due to oxidation, the more apposite and crucial experiments with the oxydases contained within the petals lend powerful support to that view.

The series of observations described in the foregoing pages lead us to the following conclusions:—

In concentrated alcohol the anthocyan pigments are reduced to the state of colourless chromogens. The reduction is brought about by reducing agents, the nature of which is unknown. The reducing agents may be specific chemical substances; they may perhaps be of the nature of catalysts; they are probably not enzymes (reductases). It is interesting to observe that an effect similar to that exercised by the reducing agent contained in stocks is brought about by hydroquinone, though not by formaldehyde.

When the concentrated alcohol is replaced by water, the oxydases, which

are not destroyed by the former reagent, resume their activity, and colourless chromogen is converted into anthocyan pigment.

The fact that the colours of the pigments thus produced are identical severally with those of the natural petals, indicates either that chromogens of more than one kind exist in the different colour-varieties of stocks, or —what for our present purpose is nearly the same thing—that one chromogen is present, and associated with it are substances which determine the coloration of the oxidised produce of the chromogen.

The petals of plants such as stocks contain much larger quantities of chromogen than are used in the natural flower. Not only may the original depth of colour be recovered, but the pigment so formed may be removed from the tissues and further instalments of pigment may be produced. Whether the reserves of chromogen contained in the flower occur as such or, as would appear more probable, in the form of prochromogen, we cannot at present say.

The factor which determines the direction in which the pigment-producing reaction shall go is the amount of active water present in the cells. As the amount of water decreases, the reducing agents of the cell become active and oxydase becomes inert; as the amount of water increases oxydase action comes into play and the reducing agents are either destroyed, or, if they persist, any action which they exert is masked by the superior and opposed activity of oxydase. The relations may be expressed diagrammatically by the following scheme:—

$$\text{Chromogen} \underset{\{-\text{Water},\ \text{Reducing agent}\}}{\overset{\{\text{Oxydase},\ +\text{Water}\}}{\rightleftharpoons}} \text{Anthocyan pigment.}$$

The occurrence of reducing bodies side by side with oxydases in the anthocyan-containing tissue of plants, the antagonistic relation which obtains between the reducing and oxidising agents of the cell, and the relations which exist between the activities of these agents and the degree of hydration of the cell are calculated to throw light, not only on the phenomena of pigment-formation and pigment-inhibition in plants, but also on others of wider import. Following the clue offered by these experiments we may hope perhaps to advance towards an understanding of the biochemical meanings of activity and latency of seeds, of the enforced and natural awakening of vegetation, and of cognate phenomena.

A discussion of the foregoing facts in relation with these phenomena lies, however, beyond the scope of the present communication.